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Targeting Sphingosine-1-Phosphate Axis in Obesity-Promoted Breast Cancer

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Richmond VA

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14. ABSTRACT

Obesity, which induces low-grade inflammation, is a known risk factor for worse prognosis in many cancers including breast. We found that sphingosine-1-phosphate (S1P) produced by sphingosine kinases (SphKs) plays a critical role in obesity-related inflammation and breast cancer. Obesity increased S1P in the tumor microenvironment, as well as in the primary tumors. FTY720, a functional antagonist of S1PR1, dramatically decreased cancer progression by reducing expressions of SphK1 and S1PR1, and inflammatory cytokines including IL-6. Our results suggest a critical role for S1P in obesity-related inflammation and FTY720, an S1P axis inhibitor, appears to be a promising treatment for breast cancer in the obese condition, could be due to its effect on reactivate ERa expression and sensitize breast cancer cells to tamoxifen therapy.

15. SUBJECT TERMS

sphingosine kinase 1, sphingosine-1-phosphate, obesity, lung metastasis, macrophage, cytokines, ER□□□inflammation

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7. Participants & Other Collaborating Organizations…… N/A

8. Special Reporting Requirements……N/A

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1. INTRODUCTION

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and anti-estrogens, such as tamoxifen, are the first line of therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to de novo or acquired resistance as well as patients with ERα, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3). Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), presenting with TNBC and endocrine therapy resistance (5). It has been proposed that chronic low grade inflammation evoked by obesity (6, 7) may play a role in aggravation of cancer progression by facilitating interactions between cancer cells and inflammatory stromal cells, such as macrophages (8, 9). Macrophage infiltration in the tumor microenvironment is recognized as an important enabler of cancer progression, and tumor associated macrophages (TAMs) correlate with increased angiogenesis, metastasis, and decreased survival of breast cancer patients (10). There is growing evidence that sphingosine-1-phosphate (S1P), a pleiotropic bioactive sphingolipid metabolite formed inside cells by two closely related sphingosine kinases, SphK1 and SphK2, is involved in inflammation and cancer (9,10). S1P regulates numerous cellular processes important for breast cancer, including cell growth, survival, invasion, lymphocyte trafficking, vascular integrity, angiogenesis, and cytokine and chemokine production, among others (9,10). Although many of the actions of S1P are mediated by 'inside-out" signaling via its receptors, designated S1PR1-5 (9), our lab has demonstrated that SphK1 and intracellular S1P also play a direct role in TNF-α signaling and the canonical NF-kB activation pathway (11), important in inflammation and cancer. We recently showed that S1P produced by upregulation of SphK1 links chronic intestinal inflammation to colitis-associated cancer (CAC) and is essential for production of IL-6, persistent activation of NF-kB and STAT3, and consequent upregulation of one of its target genes, the S1P receptor, S1PR1 (12). Our results suggest that SphK1 and S1P may also play similar roles in an animal model of breast cancer. Expression of SphK1 is elevated in patients with breast cancer (13,14) and correlates with poor prognosis (15). Further, serum S1P levels in this latter group of patients are higher than in matched controls (16). Therefore, we believe that S1P may have a critical role in obesity-related inflammation and that FTY720, an S1P axis inhibitor, could be a promising additional treatment for breast cancer in the obese condition.

2. KEYWORDS

sphingosine kinase 1, sphingosine-1-phosphate, obesity, lung metastasis, macrophage, cytokines, ERα, inflammation

3. ACCOMPLISHMENTS

The major goals of the project

Aim 1. Determine the role of SphK1 and S1P in obesity promoted low-grade chronic inflammation and tumor progression. (**Year 1**)

Aim 2. Dissect the cell-autonomous functions of the SphK1/S1P/S1PR1 axis in the primary tumor and in infiltrating myeloid cells in regulation of obesity promoted tumor progression and

metastasis. (**Year 2, 3**)

Aim 3. Targeting the SphK1/S1P/S1PR1 axis to prevent elevation of SphK1 and S1PR1 and the NF-kB /IL-6/STAT3 amplification cascade, and reactivate ER expression in ER-negative breast cancer. (**Year 2, 3**)

ACCOMPLISHED

Major activities

1. Developing the mouse models for the in vivo experiments described in Aim 1.
2. Developed reagents and mouse models that are needed for Aim 3.
3. Developed more accurate screening method for gentotyping WT, $SphK1^{-/+}$, and $SphK1^{-/-}$ pups.
4. Crossed $SphK1^{f/-}SphK2^{-/-}$ mice with transgenic mice expressing Cre recombinase under the control of the LysM promoter to generate LysM-Cre mice that specifically lack SphK1 in the myeloid lineage.
5. Established in vivo techniques including tail vein injections of cancer cells in a lung metastasis model.
6. Established the use of E0771-luc breast cancer cells in a syngeneic breast cancer model to track primary tumor progression as well as metastasis in vivo.
7. Using the new CRISPR/Cas technology for gene editing on E0771-luc cells to create stable SphK1 KO cells.
8. Developed the use of FACS as a sensitive tool for dissecting the role the SphK1/S1P axis in immune cells that participate in obesity promoted breast cancer.

Major findings

Our first goal was to determine the role of SphK1 and S1P in obesity promoted low-grade chronic inflammation and tumor progression. Because BALB/c mice are obesity resistant, in order to elucidate the effect of obesity on cancer progression and the link to SPHK1-S1P-S1PR1 axis, we used C57BL6 mice and E0771 breast cancer cells isolated from C57BL6 tumors. As expected, mice fed with high fat diet (HFD) for 20 weeks developed severe obesity with an almost 2 fold increase of body weight compared with normal diet (ND) fed mice (Fig. 1A). E0771 mouse breast cancer cells were implanted into mammary fat pad of C57Bl/6 mice, which were fed with HFD or ND for 12 weeks prior to the implantation. The mice fed with HFD developed significantly larger tumors within 30 days than those fed with ND (Fig. 1B). The proinflammatory cytokines, IL-6 and TNF-α, produced by tumor infiltrating stromal cells, such as tumor associated macrophages (TAMs), are known to have important roles in obesity-related cancer progression (10). We found that expression of these cytokines was increased in the tumors of HFD fed mice, compared to those fed with ND (Fig. 1C). Furthermore, immunofluorescent analysis with anti-F4/80 antibody, a macrophage marker, showed that tumors from HFD fed animals recruited significantly more TAMs than those from ND fed animals (Fig. 2). Since S1P links inflammation and cancer progression, and stimulates tumor-associated inflammation and increases cytokines such as IL-6 and TNF-α (28), we investigated the role of S1P in obesity-related inflammation and cancer progression. Expression of SphK1 and S1PR1, but not SphK2, was increased in the tumors from 6 mice fed with HFD (Fig. 1D). Mass spectrometry analysis revealed that while S1P levels in the normal breast mammary fat pad were increased with HFD feeding, S1P levels were even higher in breast tumors. S1P levels were also increased in the serum of the tumor-implanted animals fed with HFD compared with those fed with ND (Fig. 1F). However, minimal changes in S1P were evident in the serum of non-tumor bearing mice fed with HFD. These experiments, carried out in collaboration with Dr. Masayuki Nagahashi, a surgeon from Japan, suggest that obesity evokes inflammation, promoting secretion of S1P into the tumor microenvironment, and increasing systemic level**s**. However, it should be emphasized that this is only one experiment and we have now carried out several additional studies to confirm these results.

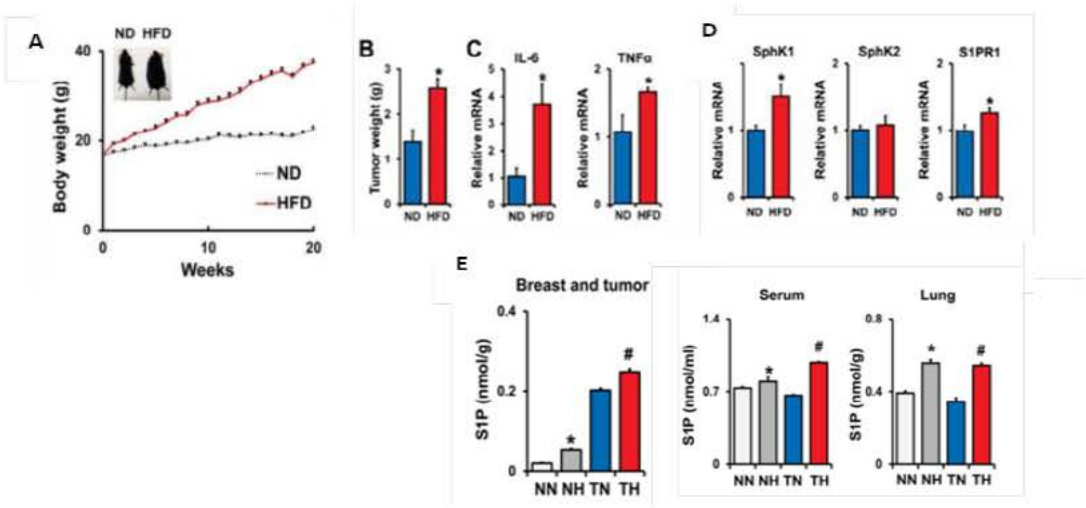

Figure 1. Effect of high fat diet on tumor growth and S1P levels. S1P in tumors and in the tumor microenvironment in E0771 syngeneic breast cancer model. A, C57Bl/6 mice were fed with normal diet (ND) or high fat diet (HFD) and body weight was measured. B, C57Bl/6 mice were fed with ND or HFD for 12 weeks, and E0771 cells were implanted into the chest mammary fat pad. Tumor weight was measured at Day 30 after the implantation. Expression of IL-6 and TNFα (C) and SphK1, SphK2, and S1PR1 (D) in E0771 tumors was determined by QPCR and normalized to levels of GAPDH mRNA. *, $P < 0.05$.

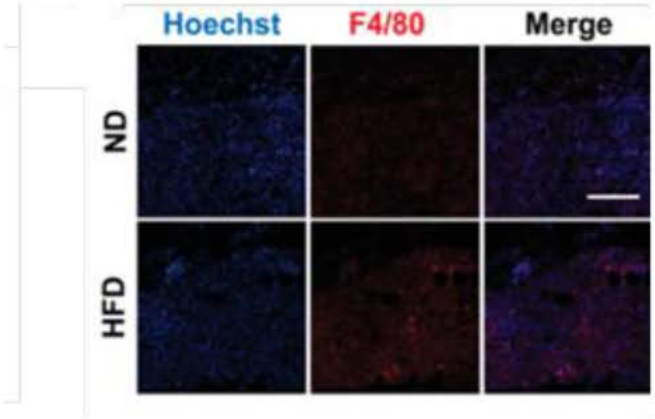

Figure 2. **Effect of high fat diet on tumor associated macrophages (TAMs)** Immunofluorescence analysis of E0771tumors from mice fed with ND or HFD at Day 30, stained with anti-F4/80 (red) and Hoechst (blue). Scale bar, 100 μm.

We also have begun targeting the SphK1/S1P/S1PR1 axis to prevent elevation of SphK1 and S1PR1 and the NF-kB /IL-6/STAT3 amplification cascade, and reactivate ER expression in ER-negative breast cancer. To this end, we have been using the 4T1 orthotopic model that closely mimics the progressive forms of estrogen-insensitive human metastatic breast cancer as a first approach. BALB/C mice were fed a high fat diet (HFD) for 12 weeks to induce obesity-related chronic low-grade inflammation. 4T1-luc2 (ER^-) murine mammary cancer cells were orthotopically implanted into the chest mammary fat pad. In this enhanced metastatic breast cancer model, we found that SphK1 expression was highly increased in the breast tumors with concomitant increases in S1P levels. Serum S1P levels in these mice were also increased, suggesting that overexpression of SphK1 in the tumor may be responsible for increased circulating levels of S1P. We also used this model to examine the importance of the SphK1-S1P-S1PR1 axis. Tumor bearing mice were treated with FTY720 which is phosphorylated in vivo to FTY720-P, a S1P mimetic that modulates the immune system by acting as a functional antagonist of S1PR1, inducing its internalization and degradation (17). FTY720 is also an

inhibitor of SphK1 (18), and induces its degradation (19,20). We also examined the notion that inhibitors that act as anti-inflammatory drugs such as FTY720 will enhance the effects of conventional hormone therapies such as tamoxifen. We used this experiment to answer part of Aim 3 as well. High expression of SphK1 and S1PR1 are also associated with development of tamoxifen resistance in ER-positive breast cancer patients. We have recently found that FTY720-P binds and potently inhibits class I HDACs (21). Therefore, as we proposed in Aim 3, we used FTY720 to target the SphK1/S1P/S1PR1 axis as a potential therapy for obesity related breast cancer and to re-express ERα both in 4T1-ERα negative cells in vitro and in vivo.

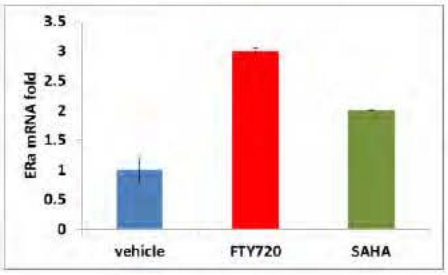

Figure 3. FTY720 treatment of ERα-negative 4T1 murine breast cancer cells induces ERα expression. Cells were treated with FTY720 (5 μM) or SAHA (1 μM) for 24 hours. ERα mRNA levels were determined by QPCR and normalized to GAPDH mRNA. *, P<0.05.

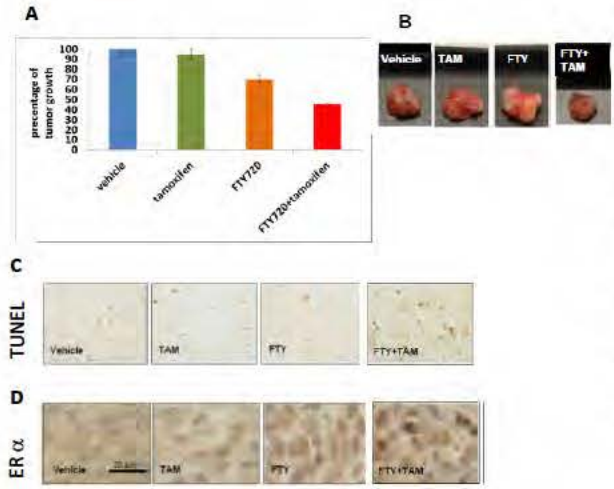

Figure 4. FTY720 reduces breast tumor growth and enhances anti-cancer effectiveness of tamoxifen in ERα negative 4T1 syngeneic xenografts. 4T1 cells were surgically implanted into the 2nd mammary fat pads. Tumor-bearing mice were randomized into groups 2 days after implantation and then treated with vehicle, FTY720 (1 mg/kg), tamoxifen (25 mg/kg), FTY720 plus tamoxifen by gavage daily till day 15. (A) Tumor volumes were measured daily. Tumor volumes and weights on day 15. (B) Representative tumors. (C,D) Immunohistochemical staining of tumor sections for TUNEL (C) and ERα (D). Scale bar: 20 μm. Quantifications of TUNEL positive cells and ERα intensity are shown. Data are mean ± SEM (n=5). *p < 0.05 compared to Vehicle

FTY720 enhanced ERα expression in 4T1 cells even more than the HDAC inhibitor, SAHA (Fig. 3). As we have found that FTY720 treatment induces functional ERα reactivation in vitro, we sought to determine whether FTY720 can also enhance anti-estrogen therapy in vivo. We utilized a syngeneic mouse metastatic breast cancer model instead of conventional xenografts in immune compromised nude mice that more accurately mimics human breast cancer (20, 38). ERα negative 4T1 cells were orthotopically implanted into the second mammary fat pad of immune-competent mice and randomized to insure similar tumor burdens prior to treatment. 4T1 cells produced large primary tumors in the chest mammary fat pad that were not significantly reduced by tamoxifen administration. Orally administered FTY720 reduced tumor growth, an effect that was significantly potentiated by co-administration of tamoxifen(Fig. 4A-B). TUNEL staining also revealed a large increase in apoptotic cells in tumors from FTY720 plus tamoxifen treated mice, compared to tumors from mice treated with each separately(Fig. 4C). In agreement with our in vitro study, we observed that nuclear expression of ERα was increased in tumors from mice that were treated with FTY720 (in the absence or presence of tamoxifen). In contrast, ERα expression was not changed in mice treated only with tamoxifen (Fig. 4D). This study suggests that FTY720 has a beneficial effect on reducing tumor growth in ER-negative breast cancer cells in combination with tamoxifen.

Conclusions:

Our initial results suggest a critical role for S1P in obesity-related inflammation. FTY720 appears to be a promising treatment for breast cancer, especially in obesity-driven breast cancer progression. FTY720 also shows promising activity as an HDAC inhibitor that can reactivate ERα expression and sensitize breast cancer cells to estrogen targeted therapies.

Opportunities for training and professional development

1. VCU's Individual Development Plans (IDPs) provides me with a planning process that identifies both professional development needs and career objectives. This plan is helping me to pursue my long-term career goals and gives me the necessary tools to meet these goals. Also it is helping me to identify short-term goals and with a clearer sense of expectations and identification of milestones along the way to achieving specific objectives.
2. I have participated in the **Southeastern Regional Lipid Conference** (SERLC), the largest lipid meeting in the US that has been held for 49 consecutive years. I have been invited to give an oral presentation and present my work.
3. I am participating in the monthly VCU **Massey Cancer Center** (MCC) seminar series, one of only 67 cancer centers designated by the National Cancer Institute, both as an attendee and speaker, as well as the annual MCC Cancer Research Retreat that was held this year in May. This annual MCC retreat gives me the opportunity to meet in person key scientists such as Dr. Lewis Cantley who discovered the PI3-kinase pathway and has had a great impact on cancer research.
4. I participated in the 2015 **American Association for Cancer Research** (AACR) annual meeting which was held in April and was a co-author of a poster on my research. This meeting not only provided me with the opportunity to establish new connections with researchers in the field but also to learn a lot about new approaches and discoveries.
5. The numerous Massey Cancer Center core facilities I extensively use for my research. For example, the flow cytometry core has been invaluable in helping me develop cell sorting methodology for our studies.

How were the results disseminated to communities of interest

We have submitted a paper on which I am a co-author.

The plan for the next reporting period

1. Using the new breeding mice colonies (WT, SphK1 KO, SphK1 specific KO in the myeloid lineage) for breast cancer models as well as for a lung metastasis model to complete Aim1 and Aim2. Moreover, that will help us to deepen our understanding of the importance of the S1P-SphK1 axis in the immune cells that participate in obesity-driven cancer progression.
2. Finish development of the E0771 SphK1 knockout cells using CRISPR/CAS and then use these cells in our breast cancer models.
3. I plan to continue the proposed research as originally suggested.

4. **IMPACT**

The impact on the development of the principal discipline of the project

Obesity, which induces low-grade inflammation, is a known risk factor for worse prognosis in many cancers including breast. We found that sphingosine-1-phosphate (S1P) produced by sphingosine kinases (SphKs) plays a critical role in obesity-related inflammation and breast cancer. Obesity increase S1P in the tumor microenvironment, as well as in the primary tumors. FTY720, a functional antagonist of S1PR1, dramatically decreased cancer progression by reducing expressions of SphK1, S1PR1, and inflammatory cytokines including IL-6. Our results suggest a critical role for S1P in obesity-related inflammation and FTY720, an S1P axis inhibitor, which act as an anti-inflammatory drug appears to be a promising treatment for breast cancer in the obese condition. Our results provide hints of a possibility that treatment with FTY720 might increase ER expression and sensitize breast cancer cells to tamoxifen therapy. This may pave the way for development of new cancer therapeutics targeting this axis as a promising strategy for effective treatment of hormonal refractory breast cancer with available anti-estrogens. These treatments are critical for the prevention of the morbidity and mortality of breast cancer, and are even more important considering the increasing rates of obesity in the US.

The impact on other disciplines

Nothing to report

The impact on technology transfer

Nothing to report

The impact on society beyond science and technology

Nothing to report

5. CHANGES/PROBLEMS

Due to a handling issue by VCU DAR staff, which did not breed the mice in the proper way, we had a problem with our mice colonies that caused us a few months setback in regards to using these mice into experimental cancer models. Luckily we caught the problem and by genotyping the entire colonies we resoled this issue and made new breeding pairs to establish the correct colonies. We are expecting to begin the experiments with these mice in the next few months.

6. PRODUCTS

1. Nitai C. Hait, **Dorit Avni**, Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Hiroaki Aoki, Catherine I. Dumur, Zara Zelenko, Emily J. Gallagher, Derek Leroith, Sheldon Milstien1, Kazuaki Takabe and Sarah Spiegel. The Phosphorylated Pro-Drug FTY720 Is a Histone Deacetylase Inhibitor that Reactivates ERα Expression and Enhances Hormonal Therapy for Breast Cancer. Oncogenesis, in press, 2015.

2. Nitai C. Hait, **Dorit Avni,** Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Sheldon Milstien, Kazuaki Takabe and Sarah Spiegel. FTY720-P Is a Potent Inhibitor of Class I Histone Deacetylases that Enhances Histone Acetylation, Reactivates ERα Expression, and Increases Hormonal Therapeutic Sensitivity of Breast Cancer. AACR abstract, April 18-22, 2015, Philadelphia, PA.

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APPENDICES

CURRICULUN VITAE

NAME DORIT AVNI	POSITION TITLE POSTDOC

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Tel Aviv University	BSc	1997	Biochemistry
Tel Aviv University	MS	2000	Cardiology
Tel Aviv University	PhD	2010	Immunology

Employment Experience

2012-present: Post-doctoral training, lab of Dr. Sarah Spiegel, Department of Biochemistry & Molecular Biology, Virginia Commonwealth University, Richmond Virginia

2011: Post-doctoral training, lab of Dr. Tsaffrir Zor, Biochemistry & Molecular Biology Department, Tel Aviv University

2008-2012: Scientific consultant, Allosterix-pharma Ltd., Yozmot Incubator, Emek-Hefer.

2005-2010: Ph.D. Research, lab of Dr. Tsaffrir Zor, Biochemistry and Molecular Biology Department, Tel Aviv University

2005-2011: Supervision of 8 undergraduate (project) students and 3 M.Sc. students

2005-2009: Teaching Assistant, Faculty of Life Sciences, Tel Aviv University

2003-2004: Research Assistant, ImmunoBar Ltd., Weizmann Institute (Rehuvot) + Sourasky Tel-Aviv medical center (Tel-Aviv). Establishment of a new biotech start-up as well as research focused on therapy for colon cancer.

2000-2002: Research Assistant, Prochon Biotech Ltd., Rehovot. My research focused on identifying and testing treatments for skeletal diseases and cancer

1997-2000: M.Sc. Research, lab of Prof. Uri Oron, Faculty of Life Sciences, Tel Aviv University

1997-1999: Teaching Assistant and supervision of undergraduate students, Tel Aviv University

1996-1997: Research Assistant, undergraduate project student, lab of Prof. Uri Oron, Faculty of Life Sciences, TAU. Clinical research in the field of cardiosurgery, using biochemical and statistical analysis.

Scholarships

2011: FEBS Scholarship for presentation in the 36th congress of the European Biochemical Societies, Torino, Italy

Conference Presentations

2015: Cancer research retreat, Massey Cancer Center, VA, USA
2015: Annual meeting of American Association for Cancer Research (AACR)
2014: Southeastern Regional Lipid Conference (SERLC)
2014: Cancer research retreat, Massey Cancer Center, VA, USA
2013: Cancer research retreat, Massey Cancer Center, VA, USA
2012: Cancer research retreat, Massey Cancer Center, VA, USA.
2011: The 36th congress of the European Biochemical Societies (FEBS), Torino, Italy
2011: The 6th congress of the federation of the Israel Societies for Experimental Biology (FISEB) Eilat, Israel.
2010: The Annual Meeting of the Israel Society for Biochemistry and Molecular Biology. Rehovot, Israel.
2009: Israeli Immunological Society 37th Annual Meeting.
2008: The 5th congress of the federation of the Israel Societies for Experimental Biology (FISEB) Eilat, Israel.
2004: Annual Meeting of the Israel Society for Biochemistry and Molecular Biology Tel-Aviv, Israel
2000: The congress of the Israel society of anti-oxidants, Bar-Ilan, Israel

Professional Associations

2012-present: Membership in American Association for Cancer Research
2004-2012: Membership in the Israel Biochemistry and Molecular Biology Association

Publications

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